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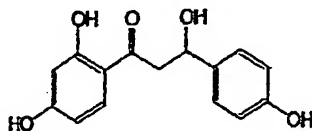
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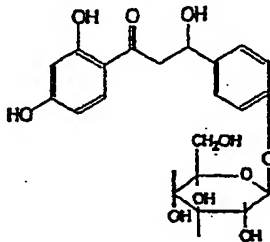
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(54) Title: ANTI-CARCINOGENIC ACTIVITY OF HYDROXYLATED CHALCONE COMPOUNDS EXTRACTED FROM LICORICE ROOT

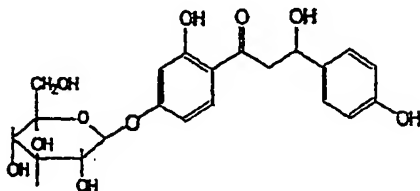
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(57) Abstract: Compositions and methods are provided for prevention and treatment of cancer. The compositions comprise pure hydroxylated chalcone compounds of licorice root (*Glycyrrhiza glabra*) including 1-propanone-1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4'-hydroxyphenyl).

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ANTI-CARCINOGENIC ACTIVITY OF HYDROXYLATED CHALCONE
COMPOUNDS EXTRACTED FROM LICORICE ROOT

Introduction

This application claims the benefit of priority from
5 Provisional Application Serial No. 60/211,266 filed June 13,
2000.

Background of the Invention

Herbal products have gained popularity for their use in
the treatment of diseases in humans. Although the clinical
10 effect of most herbal products is unknown, many herbs contain
derivatives with biological activity. One such herb is
licorice root. Extracts of licorice root have been shown to
have biological activity that includes antioxidant activity
(Palagina, M.V. et al. 1999. *Ter. Arkh.* 71:45-48), inhibition
15 of melanin synthesis (Yokota, T. et al. 1998. *Pigment Cell*
Res. 11:355-361), inhibition of angiogenesis (Kobayashi, S.
et al. 1995. *Biol. Phar., Bull.* 18:1382-1386), anti-microbial
activity (Mitscher, L.A. et al. 1980. *J. Nat. Prod.* 43:259-
269), anti-parasitic activity (Zhai, L. et al. 1995.
20 *Antimicrob. Agents Chemotherap.* 39:2742-2748), and anti-tumor
activity (Shibata, S. 1994. *Stem Cells* 12:44-52). Several
compounds responsible for the various biological effects have
been isolated. Examples of such compounds include glabridin
(Yokota, T. et al. 1998. *Pigment Cell Res.* 11:355-361),
25 isoliquiritin (Kobayashi, S. et al. 1995. *Biol. Pharm. Bull.*
18:1382-1386), glycyrrhizin (Raggi, M.A. et al. 1995. *Boll.*
Chim. Farm. 134:634-638), and licochalcone A, a non-
hydroxylated chalcone compound (Shibata, S. 1994. *Stem Cells*
12:44-52).

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Recent studies with a combination of eight herbs, that included licorice root, called PC-SPES, has been shown to have potent clinical and biological activity (DiPaola, R.S. et al. 1998. *N. Engl. J. Med.* 339:785-791). PC-SPES showed anti-
5 prostate cancer activity which was attributable to phytoestrogens that produced a chemical castration. Another study demonstrated that licorice root alone was capable of decreasing circulating testosterone levels in men (Armanini, D. et al. 1999. *N. Engl. J. Med.* 341:1158). Additional
10 studies in patients have demonstrated PC-SPES to have anti-tumor activity refractory to chemical castration, thus indicating that other mechanisms may be responsible for the anti-tumorigenic activity of this licorice root-herbal combination therapy (Small, E. et al. 1999. *N. Engl. J. Med.*
15 340(7):785-791).

PC-SPES extracts have also been shown to induce apoptosis in tumor cell lines and decreased the expression of bcl-2. Bcl-2 is a 26 kDa protein that blocks cell death by inhibiting cytochrome c release from mitochondria, a critical
20 event in the apoptotic pathway. Overexpression of bcl-2 protects cells from death promoting stimuli, whereas lowering bcl-2 levels increases cell death and sensitivity to chemotherapy (Reed, J.C. 1997. *Nature* 387:773-776). Recent
studies suggest that drugs which decrease bcl-2 expression,
25 or inactivate the molecule through phosphorylation, induce apoptosis. For example, paclitaxel, docetaxol, vincristine, and vinblastine alter microtubule structure and induce apoptosis in association with bcl-2 phosphorylation (Hadlar, S. et al. 1996. *Cancer Res.* 56:1253; Haldar, S. et al. 1995.
30 *Proc. Natl. Acad. Sci. USA* 92:4507-4511).

It has now been found that compounds extracted from licorice root, in particular hydroxylated chalcones, have activity consistent with induction of apoptosis and potential activity as anti-tumorigenic and anti-carcinogenic agents.

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Summary of the Invention

An object of the present invention is to provide a hydroxylated chalcone compound extracted and purified from *Glycyrrhiza glabra*. In a preferred embodiment the compound
5 comprises 1-propanone-1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4'-hydroxyphenyl).

Another object of the present invention is to provide a method of inducing phosphorylation of bcl-2 comprising contacting cells or tissues with a hydroxylated chalcone
10 compound.

Yet another object of the present invention is to provide a method of inducing apoptosis in cells or tissues comprising contacting cells or tissues with a hydroxylated chalcone compound.

15 Also included in the present invention are methods for inhibiting tumor cell growth and preventing and treating cancer via contacting tumor cells or tissues with an effective amount of a hydroxylated chalcone compound.

Description of the Drawings

20 Figure 1 depicts structures of several hydroxylated chalcones of the present invention that were identified by mass spectrometry and NMR. Figure 1A is the structure of the parent compound, 1-propanone-1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4'-hydroxyphenyl). Figure 1B depicts a
25 glycosylated derivative of the parent compound which is referred to herein as 1-propanone-1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4'-hydroxyphenyl-4'-o-beta-D-glucopyranoside). Figure 1C is a second glycosylated derivative of the parent compound which is referred to herein as 1-propanone-1-(2,4-
30 dihydroxyphenyl-4'-o-beta-D-glucopyranoside)-3-hydroxy-3-(4'-hydroxyphenyl).

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Detailed Description of the Invention

A specific component of licorice root extract, a hydroxylated chalcone, has now been identified which has biological activity consistent with anti-tumorigenic effects in animals, including humans. It is believed that this licorice root extract component can be used as an anti-cancer agent in the prevention and treatment of cancer in animals, including humans.

The hydroxylated chalcones of the present invention were identified by extracting licorice root with methanol, ethanol, DMSO or ethyl acetate. Crude extract fractions were collected and the effects of various fractions of whole licorice root were assessed by immunoblotting. Licorice root extracted with ethyl acetate, DMSO, or ethanol induced bcl-2 phosphorylation as demonstrated by a slower migrating band as compared to the vehicle control (ethanol alone) or a water extract.

Previous studies have confirmed an association between bcl-2 phosphorylation and cell-cycle arrest at G2/M. Accordingly, the effects of the various licorice root extracts on cell cycle were also assessed. Licorice root extract induced G2/M cell cycle arrest in a similar manner to paclitaxel (control). Thus, these results demonstrate that the licorice root extract has biological activity similar to known anti-microtubule drugs.

To identify the active component in the licorice root extract capable of bcl-2 phosphorylation, fractions were collected and assessed by HPLC. The extract contained multiple derivatives. Accordingly, the focus was placed on the three major peaks determined by HPLC. Fractions eluted from major peaks 1, 2, and 3 were shown to induce bcl-2 phosphorylation in a manner similar to paclitaxel-treated controls. Analysis by NMR and mass spectrometry revealed that peak 3 contained a hydroxylated chalcone compound (referred to herein as DC and depicted in Figure 1A). Peaks 1 and 2 were two glycosylated derivatives of DC (Figures 1B and 1C,

respectively). Other polyphenol structures found in various foods, such as resveratrol, an estrogenic compound isolated from red wine (Goldberg, D.M. et al. 1996. *Am. Jour. of Enol. Vitic.* 47:415-420), have been suggested as potential anti-
5 cancer and chemopreventative agents. A DC-type compound has also been isolated from another natural product, *Rosa cymosa* (Yoshida et al. 1993. *Phytochemistry* 32:1033-1036), However, the biological activity of this DC-type compound has not been determined.

10 The activity of purified DC was determined in additional testing. DC was shown to induce phosphorylation of bcl-2 in both MCF-7 and DUPro-1 tumor cells. In addition, the pure DC induced G2/M cell cycle arrest similar to whole licorice root extracts. In these experiments MCF-7 tumor
15 cells were treated with DC and assessed by flow cytometry. DC induced a G2/M cell cycle arrest in a manner similar to the known anti-microtubule agent paclitaxel. However, DC was shown to induce microtubule fragmentation in a manner similar to vinblastine, while paclitaxel has been shown to induce
20 microtubule bundles. Therefore, DC is actually a microtubule destabilizer, more similar to vinblastine. These data demonstrate that a hydroxylated chalcone compound with no methoxy groups still has significant anti-microtubule activity similar to chalcone structures with multiple methoxy groups
25 (Edwards, M.L. et al. 1990. *J. Med. Chem.* 33:1948-1954).

The cytotoxicity of DC was then assessed in an apoptosis assay. In this assay, tumor cells were treated with pure DC and cell viability and apoptosis responses were assessed. DC induced apoptosis in MCF-7 cells, as demonstrated by the
30 detection of extracellular phosphatidylserine, which redistributes to the outer layer of the membrane during apoptosis. Early apoptotic cells demonstrated green fluorescence under microscopy. Necrotic cells were identified by their yellow-red intracellular staining appearance. DC
35 induced apoptosis in a manner similar to that of 10 μ M

camptothecin (control compound). DC also decreased cell viability in MCF-7 cells in a dose-dependent manner (IC_{50} of 13 μM).

These biological activity data demonstrate the a specific licorice root extract, DC, has biological activity that is indicative of potential anti-tumorigenic effects in humans. Specifically, DC induces apoptosis and bcl-2 phosphorylation.

Thus, the present invention relates to compositions comprising a pure hydroxylated chalcone compound of *Glycyrrhiza glabra*. These compounds may be extracted and purified from *Glycyrrhiza glabra*. Alternatively, the hydroxylated chalcone compounds can be prepared synthetically using methods well known to those skilled in the art. Further, one of skill in the art can now develop new compounds with similar structure and activity to the hydroxylated chalcone compounds of the present invention based on routine methods for testing of potential clinical compounds. Compositions of the present invention preferably further comprise an acceptable pharmaceutical vehicle for administration of the pure hydroxylated chalcone compound. Selection of acceptable pharmaceutical vehicles is performed routinely by those skill in the art and multiple formulation examples are provided in standard text references such as *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985.

As demonstrated herein, compositions comprising a pure hydroxylated chalcone compound of *Glycyrrhiza glabra* induce bcl-2 phosphorylation in tissues and cells, in particular tumor cells or tissues, from animals, including humans. Compositions of the present invention are also useful in the induction of apoptosis in cells or tissues, in particular tumor cells or tissues, from animals, including humans. Thus, the compositions of the present invention are believed to be useful in methods for the prevention and treatment of cancer

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in animals, including humans. Accordingly, the present invention also relates to methods for prevention and treatment of cancer and tumor cell growth in animals, including humans, which comprises administering to the animal an effective amount of a composition containing a pure hydroxylated chalcone compound of *Glycyrrhiza glabra*. In the context of the present invention, by "effective amount" it is meant an amount of a pure hydroxylated chalcone compound of *Glycyrrhiza glabra* capable of producing a pharmacological response including, but limited to, induction of bcl-2 phosphorylation, induction of apoptosis, inhibition of tumorigenesis, or prevention or treatment of cancer. Effective amounts of the compounds to be administered can be determined routinely by those of skill in the art based upon pharmacological response data such as that provided herein. For example, doses to be administered are routinely determined by those skilled in the art based upon data from *in vitro* assays such as IC₅₀ determinations as provided in the instant application. Routes of administration, as well as dosing regimes, can also be determined routinely by one of skill in the art based upon prior experience with similar compounds, such as resveratrol.

The following non-limiting examples are presented to better illustrate the present invention.

EXAMPLES

Example 1: Extraction and Isolation of Licorice Root Compounds

Powdered roots of *Glycyrrhiza glabra* were extracted with methanol and concentrated under vacuum using rotary evaporation (Rotavapor R-110, Buchi, Switzerland). The remaining concentrate was then partitioned with acidified ethyl acetate (3% HCL). The dry ethyl acetate extract was then chromatographed on a reversed phase octadecyl-functionalized silica gel column such that bio-assay directed

fractionation could be performed. Elution was done using a solvent mixture of water/methanol with an increasing concentration of methanol (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100; each 500 ml). Successive 5 fractions were collected and tested for biological activity.

The most active fraction was re-chromatographed on a semi-preparative Zorbax Rx-C18 reversed phase HPLC column (9.4 mm x 240 mm, 5 μ m) purchased from Mac-Mod Analytical (Chadds Ford, PA). Compounds were eluted by a gradient solvent system 10 (A: water and 0.05% formic acid; B: acetonitrile). The elution program at 3 ml/min was as follows: 80% A to 40% B (0 to 45 minutes). The wavelength monitored was 254 nm. Successive fractions were collected and sent for additional biologic testing.

15 Fractions were screened for purity using a Discovery C18 reversed phase HPLC column (250 mm x 4.6 mm; 5 μ m) with a column guard purchased from Supelco (Bellefonte, PA). The solvent program was a gradient system (A: water and 0.05% formic acid, B: 100% acetonitrile; 35 to 55 minutes). The 20 elution program at 1 ml/minute was as follows: 100% A to 100% B (0 to 35 minutes); 100% B (35 to 55 minutes). The wavelengths monitored were 220 to 320 nm with a Varian 9065 diode array detector. Final separation of pure compounds was obtained using a semi-preparative HPLC on a Zorbax Rx-C18 25 reversed phase column (9.4 mm x 240 mm, 5 μ m) purchased from Mac-Mod Analytical. Compounds were eluted by a isocratic solvent system containing 82% water with 0.05% formic acid, 18% acetonitrile.

Example 2: Compound Identification

30 Both ^1H and ^{13}C NMR spectra were obtained on a VXR-200 instrument. Mass spectrometry was performed on a Micromass Platform II system equipped with a Digital DECPc XL560 computer for analysis of data. Mass spectra were obtained using atmospheric pressure chemical ionization (APCI) in the

negative-ion mode. The ion source temperature was set at 150 C and the probe was set at 450 C. The sample cone voltage was 10 V and the corona discharge was 3.2 kV. HPLC analysis was performed on a Varian Vista 5500 liquid chromatograph pump
5 coupled to a Varian 9065 Polychrom diode array detector. Semi-preparative fractionation of purified compounds was obtained on a Varian 9012 HPLC pump coupled to a Waters Lambda-Max Model 481 LC spectrophotometer. Octadecyl-Functionalized silica gel (60A particle size) was used for
10 column chromatography. The column packing was purchased from Aldrich Chemical Company (Milwaukee, WI). All solvents used for extraction and isolation were of HPLC grade.

Example 3: Bcl-2 Expression and Phosphorylation Assay

Analysis of bcl-2 protein expression was performed using
15 a Western blot assay as previously described (Halдар, S. et al. 1996. *Cancer Res.* 56:1253; Halдар, S. et al. 1995. *Proc. Natl. Acad. Sci. USA* 92:4507-4511). Protein identification was made using a monoclonal bcl-2 primary antibody (DAKO Corporation) and secondary goat anti-mouse horseradish
20 peroxidase conjugated antibody (Bio-Rad Laboratories, Richmond, CA). The phosphorylation of bcl-2 was determined by mobility shifts in the Western bolt as described by Halдар (Halдар, S. et al. 1996. *Cancer Res.* 56:1253; Halдар, S. et al. 1995. *Proc. Natl. Acad. Sci. USA* 92:4507-4511).

25 Example 4: Cell Cycle Analysis

Cells were treated for 24 hours, incubated with 10 μ M BrdU for 45 minutes at 37°C. Cells were then washed with ice-cold PBS, resuspended in 200 μ l PBS and fixed with cold 70% ethanol. The cells were resuspended, incubated for 30 minutes
30 in 2 N HCl/0.5% Triton X-100 in PBS, and neutralized by rinsing once in 0.1 M sodium tetraborate (pH 8.5). Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU

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antibody (Becton Dickinson) was added (10 μ g/sample) in 50 μ l of 0.5% Tween 20/ 1% BSA in PBS and incubated for 30 minutes. The cells were washed and resuspended in 1 ml of PBS containing 5 μ g/ml propidium iodide. Fluorescence intensity was determined by quantitative flow cytometry and profiles were generated on a Becton Dickinson FACScan. A minimum of 10,000 cells were analyzed.

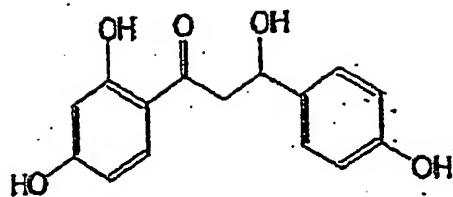
Example 5: Cell Viability and Apoptosis Assay

The Apoalert Annexin V-EGFP method (CLONTECH, Palo Alto, CA) was used to assess for apoptosis. Briefly, tumor cells were treated for 2 hours and cells were washed with fixing solution and stained with Annexin V-EGFP and propidium iodide for 15 minutes in the dark. Cells were viewed using a Nikon Eclipse TE-200 (Nikon Corporation, Tokyo, Japan) inverted fluorescent microscope. Photographs were captured using a SPOT digital camera (Diagnostic, Inc., Sterling Heights, MI) in combination with SPOT labeling with an APO-BRDU kit (PharMingen, San Diego, CA). Cells (1×10^6 per dish) were treated for 12 hours, washed with PBS, and fixed in 1% paraformaldehyde in ice for 30 minutes. After fixation, cells were washed twice with PBS and fixed in 70% ethanol. The pellets were washed and resuspended in 50 μ l of the DNA labeling solution containing Br-dUTP and TdT enzyme and incubated for 60 minutes at 37°C. After incubation, the pellets were washed, incubated with FITC labeled anti-BrdU antibody in the dark for 30 minutes at room temperature, and stained with propidium iodide and RNase. The stained cells were analyzed by flow cytometry after 30 minutes. Cell viability was assessed by the tetrazolium dye method as previously described (Scudiero, D.A. et al. 1988. *Cancer Res.* 48:4827-4833). Cells were plated in 96 well plates and incubated with various agents for 72 hours. Absorbance was measured at 570 nm using a Dyatech microplate reader.

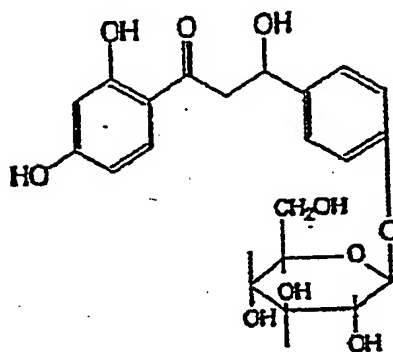
What is claimed is:

1. A composition comprising a pure hydroxylated chalcone compound of *Glycyrrhiza glabra*.
2. The composition of claim 1 wherein the compound is
5 1-propanone-1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4'-hydroxyphenyl).
3. A method of inducing phosphorylation of bcl-2 in cells or tissues comprising contacting cells or tissues with the composition of claim 1.
- 10 4. A method of inducing apoptosis in cells or tissues comprising contacting cells or tissues with the composition of claim 1.
5. A method of inhibiting growth of tumor cells comprising contacting tumor cells with the composition of
15 claim 1.
6. A method of preventing or treating cancer or tumor cell growth in an animal comprising administering to the animal the composition of claim 1.

A



B



C

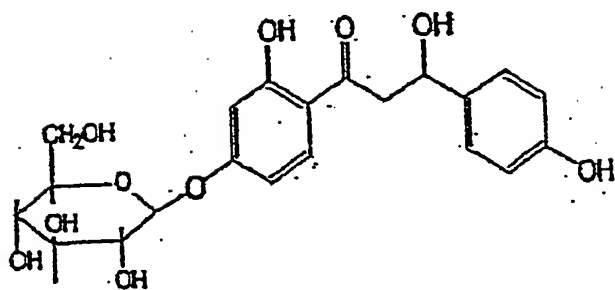


FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/19019

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 35/78

US CL : 424/725, 773

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/725, 773

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHIBATA, Anti-tumorigenic Chalcones, Stem Cells 1994, Vol.12, pages 44 - 52, especially abstract and introduction	1, 5 - 6
X, P	RAFI et al, Modulation of bcl-2 and Cytotoxicity by Licochalcone-A, a Novel Estrogenic Flavonoid, Anticancer Research 2000, Vol.20, No.4, pages 2653 - 2658, especially	1, 3 - 6
X	RAFI et al, Licochalcone-A: A Novel Phytoestrogen with Antitumor Activity in Breast and Prostate Tumor Cell Lines, Proc. Annu. Meet. Am. Soc. Clin. Oncol. 1999, Vol.18, page A 712	1, 5 - 6
A	JP 02-204495 A (MARUZEN KASEI CO LTD) 14 August 1990 (14.28.90), abstract	1 - 2
A	COMBEST, Herbal Pharmacy: Licorice, US Pharmacist April 1998, Vol.23, No.4, see entire document	1 - 6

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

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Continuation of B. FIELDS SEARCHED Item 3:

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search terms: licorice, Glycyrrhiza glabra, bcl-2, cancer, tumor, apoptosis, chalcone, licochalcone

MEDLINE

search terms: licorice, Glycyrrhiza, chalcone, licochalcone, tumor, cancer, cell growth, apoptosis